Alteration in Plasma Testosterone Levels in Male Mice Lacking Soluble Epoxide Hydrolase

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Supplemental Experimental Procedure

Oxylipin extraction and analysis

Oxylipins were extracted from plasma samples by solid phase extraction and analyzed by LC-MS/MS as described previously (5). Concentration of plasma oxylipins was normalized based on the total plasma volume collected from each animal. Analytes were quantified using standard methods (1), and five point calibration curves fit with 1/x weighted quadratic curves. Internal standard (1-cyclohexylureido,3;dodecanoic acid ,CUDA) and surrogates used in this study were as previously described (1). Surrogate recovery of 70% and up was accepted for this study.

Anxiety-related behavior

Open field: exploratory activity was assessed in the open field and recorded by video camera. Mice were placed in the open field arena (40 x 40 x 30 cm) with a 16-square grid clean floor (2). Murine behavior and activity were monitored for five minutes (6). Activity was determined by the number of lines each mouse crossed during five-minute period. Grooming, rearing, head bobbing, urination and hunched body posture all were recorded during this time (1, 3, 4).

Hedonic response

Wild-type and knockout male mice were first trained to consume a pre-warmed sweetened condensed milk solution (5%). Training consisted of an initial daily short exposure to sweetened solution instead of water in their home cage at the same time for five minutes over a weeklong period. At the end of the training period, the sweetened solution was introduced again for 20 minutes at the same time for additional seven more days. The mice were recorded with a video camera during these studies. The videos were examined for any unusual behavior (1, 3).

Total sterols

To 50 µl of plasma samples, a 10 µl mixture of deuterated surrogates was added for recovery assessment. Ethanolic potassium hydroxide (250 µl, 1 M) was added to all samples. Microtubes were purged with nitrogen, capped and vortexed for 30 seconds. Each sample was then incubated at 70°C for 1 hour, cooled for 15 minutes (4°C) and 250 µl deionized water was added. The free sterols were then extracted with two 500 µl volumes of hexane:ethanol (20:1). The combined hexane layers were transferred to a GC vial, dried down under nitrogen and reconstituted in 50 µl decane. To each sample, 30 µl Tri-Sil derivatizing reagent was added, which was then strongly mixed for 10 seconds. Finally, silylated sterols were injected onto an 6890/5975 GC/MS (Agilent Technologies, CA) with an 30 mm x 0.25 mm Rxi-5 ms column (Restek, PA) with helium as the carrier gas. Mass spectrometric analysis was performed in the single ion monitoring (SIM) mode with electron ionization.

Data analysis and statistics

Results were tested for statistical significance using *t* test or Student-Newman-Keuls test after one-way repeated measures ANOVA. Differences were considered significant if the *p* value was less than 0.05.

Supplemental Results

Male genitalia

Seminal vesicles and testes from both *EPHX2*-null and wild-type mice are presented in Figure S1. Testis measurements showed a remarkable difference between the two genotypes (Fig. S1). In both age populations examined (12-16 and 26-30 weeks of age) testis size and weight were significantly lower for the sEH-null mice (Fig. S1A), while no significant differences in mean body weight was observed between wild-type and sEH-deficient mice (Main Text, Fig. 1A). In age-matched animals, the size of the testis was smaller among the *EPHX2*-null mice (Fig. S1A). Seminal vesicle size is also slightly smaller in male mice lacking expression of sEH (Fig. S1B). Development of male accessory organs as well as testis development is regulated by testosterone. These data indicate that *EPHX2*-null male mice have impaired androgen levels.

Plasma lipids

Plasma oxylipin concentrations from both female and male of *Ephx2*-null and wild-type mice are shown in Table S1 and Figure S2. Compared with their wild-type counterparts, both female and male null mice showed elevated ratios of plasma linoleate and arachidonate epoxides to their vicinal diols 12,13-EpOME:DiHOME and 8,9- EET:DHET (Figure S2 A and B). Surprisingly, arachidonate epoxide fatty acids levels were slightly lower in female null-mice compared to the female WT animals (Table S1), while diols of both arachidonic acid (AA) and linoleic acid (LA) were elevated in the WT females (Table S1). That could be explained by the temporal monthly hormonal changes occuring in females. Nevertheless, the ratio of epoxides to diols was significantly higher in *Ephx2*-null male and female mice (Table S1 and Figure S2), indicating that gene disruption of sEH affects plasma levels of epoxides and diols of LA and AA. Interestingly, cyclooxygenase metabolites, TXB2, PGF2α and PGE2 were elevated in plasma samples of WT mice (Table S1). When looking at the global fatty acid content, the overall decrease in lipid content is mainly driven by lower levels of total PUFA and n-6 fatty acids in sEH-KO plasma samples, but the n-6 to n-3 fatty acid ratio was 4.2 for both genotypes (Table S2).

Anxiety-related behavior

Open field activity is shown in Movie S1. It became apparent that the wild-type mice were more active and start to move and examine their environment within a few seconds from the time they were placed in the new environment (Movie S1 A), whereas, the null mice were more hesitant. On average, it took the null mice more time to initiate exploring behavior (started to move around three minutes after placement, Movie S1B). General behavior such as grooming or mounting was not notably different. Both male WT and null mice were also recorded as they consumed sweetened solutions in their home cage. Evidently, the WT mice showed more enthusiasm and consumed more solution than the *Ephx2*-null mice (Movies S2A and B), while the null mice showed less enthusiasm toward the treat (Movie S2C). It is also interesting that the WT-mice showed an active social behavior that was represented by alternating the drinking time between each other. In one case, the two mice apparently tried to cover the source of sweet liquid with the cage bedding and soak it for later use (Movie S2B). Although it is not clear what the reason of this behavior is, the sEH-deficient mice show a different behavior than their WT counterparts.

Fatty acid side-chain composition was further analyzed for each lipid class (Fig. S3). The reduction in esterified cholesterol is associated with a significant decrease of n-3 and n-6 polyunsaturated fatty acid chains (Fig. S3 A). This separation was mainly driven by a decrease of linoleic acid, arachidonic acid and α–linolenic acid (18:2n6, 20:4n6 and 18:3n3, respectively). A slight but insignificant increase was observed in free fatty acid (FFA) levels in null plasma samples (Fig. S3 B). While TAG levels were reduced in the sEH-KO plasma samples, the component composition of fatty acids of both TAG and its product, DAG show insignificant differences (Fig. S3, C and D). Phosphatidylcholine was significantly decreased in sEH-KO plasma samples (S3 F).

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Table S1. Plasma oxylipin concentration (nM) determined in male and female of *Ephx2* wild-type (*Ephx2*$^{+/+}$) and null (*Ephx2*$^{-/-}$) mice.

	Ephx2$^{+/+}$		*Ephx2*$^{-/-}$	
Analyte (nM)	**Female**	**Male**	**Female**	**Male**
Epoxygenase metabolism				
12(13)-EpOME	26±3	37±1	59±5 [a]	69±8 [b]
9(10)-EpOME	21±0	31±4	25±2	33±5
14(15)-EET	10±3	3±1	6±1	5±0
11(12)-EET	8±1	4±2	5±0 [a]	7±2
8(9)-EET	8±2	3±1	7±2	7±2
5(6)-EET	47±17	16±13	37±31	96±26 [b]
Soluble epoxide hydrolase metabolism				
12,13-DHOME	19±2	44±2	7±0 [a]	11±1 [b]
9,10-DHOME	50±3	58±5	44±1 [a]	59±1
14,15-DHET	2±1	2±0	1±0 [a]	1±0 [b]
11,12-DHET	2±1	2±1	1±0	1±0
8,9-DHET	20±3	14±2	11±4	13±3
5,6-DHET	4±0	2±0	2±0 [a]	3±0
ω-Hydroxylase metabolism				
19-HETE	10±1	4±1	4±2 [a]	5±2
20-HETE	4±3	4±0	7±5	3±1
Lypoxygenase metabolism				
13-HODE	525±393	246±65	76±8	148±29
9-HODE	105±32	115±11	44±2	74±7 [b]
15-HETE	45±24	19±3	9±1	18±2
11-HETE	18±6	14±1	5±0 [a]	8±2 [b]
8-HETE	205±92	95±36	80±69	59±48
5-HETE	18±5	13±1	12±0	12±1
Cyclooxygenase metabolism				
TXB2	27±13	27±0	7±3 [a]	6±1 [b]
6-keto-PGF1α	162±96	62±21	14±4	57±34
PGF2 α	34±12	11±2	11±5 [a]	5±1
PGE2	6±3	2±1	1±0	1±0
PGD2	6±3	3±1	1±0	1±0
PGB2/PBJ2	6±0	8±4	1±1 [a]	3±1
15-deoxy PGJ2	14±8	2±0	3±3	2±2
LTB4	3±1	2±0	1±0	2±0

[a] $p \leq 0.05$ versus female wild-type; [b] $p \leq 0.05$ versus male wild-type (tailed *t* test).

Table S2. Plasma lipid content in wild-type (*Ephx2*$^{+/+}$) and *Ephx2*-null (*Ephx2*$^{-/-}$) mice

	***Ephx2*$^{+/+}$**	***Ephx2*$^{-/-}$**
Total Fatty Acid	10,300 ± 560	7,880 ± 1040
Total SFA	3,630 ± 200	2,810 ± 370
Total MUFA	1,690 ± 160	1,330 ± 130
Total PUFA	4,990 ± 230	3,720 ± 540 *
Total n3	970 ± 60	710 ± 110
Total n6	4,020 ± 170	3,000 ± 430 *
Total n7	310 ± 50	240 ± 40
Total n9	1,380 ± 120	1,100 ± 100
Total dimethoxy acetal	34 ± 2	23 ± 3 *

All values are expressed as the mean (nmol/g) ± SEM. SFA indicates saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. *, $p \leq 0.05$, *t* test, n=4-5.

FIGURE LEGENDS

Figure S1. Representative photographs of testes and seminal vesicles. **A.** Testes dissected from sEH-WT and null mice were ranged from males three or eight months of age (right and left panel, respectively). sEH-null mice show undersized testes. The paired upper and lower photographs represent multiple replicates taken at different times. **B.** View of dissected adult male *EPHX2*-wild-type and null seminal vesicles. All photos were taking with a digital camera where WT and null testes were placed side by side for a comparison. Square dimensions were 11 x 11 mm for x and y axis.

Figure S2. Plasma oxylipin profile in male and female *Ephx2*-WT or null mice. Plasma sample from female **(A)** or male **(B)** of each genotype was extracted and subjected to LC/MSMS analysis as described in Supplemental Experimental Procedure. Data are presented as AVG ± SEM of ratio of epoxides of linoleate and arachidonate to their vicinal diols of N=4 (* $p < 0.05$, # $p < 0.1$ *t* test). EpOME, epoxyoctadecenoic acid; DHOME, dihydroxyoctadecenoic acid; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid.

Figure S3. Fatty acid side-chain composition of various lipids in plasma samples of *Ephx2*-null and WT male mice. Plasma samples of WT (n=5) and null (n=4) sEH were subjected to lipid analysis. Values are presented as means of plasma lipid concentration ± SEM of **A.** cholesterol esters, **B.** free fatty acids, **C.** triacylglycerol, **D.** diacylglycerol, **E.** phosphatidylethanolamine, **F.** phosphatidylcholine, **G.** lysophosphatidylcholine and **H.** sphingomyelin. * $p < 0.05$ *t* test. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid and dm, dimethoxy acetal. *, $p \leq 0.05$ (*t* test, n=4-5).

Movie S1. Open field activity. Mice were placed in the open field arena (39 x 39 x 30 cm) a 16-square grid clean floor. Activity was monitored for five minutes and recorded on a video camera for *Ephx2*-WT **(A)**, and *Ephx2*-null mice **(B)** as described under Supplemental Experimental Procedure.

Movie S2. Sweetened condensed milk solution intake was recorded in wild-types **(A** and **B)** or knockout **(C)** male mice in their home cage for five minutes for further behavior analysis.

Figure S1 A.

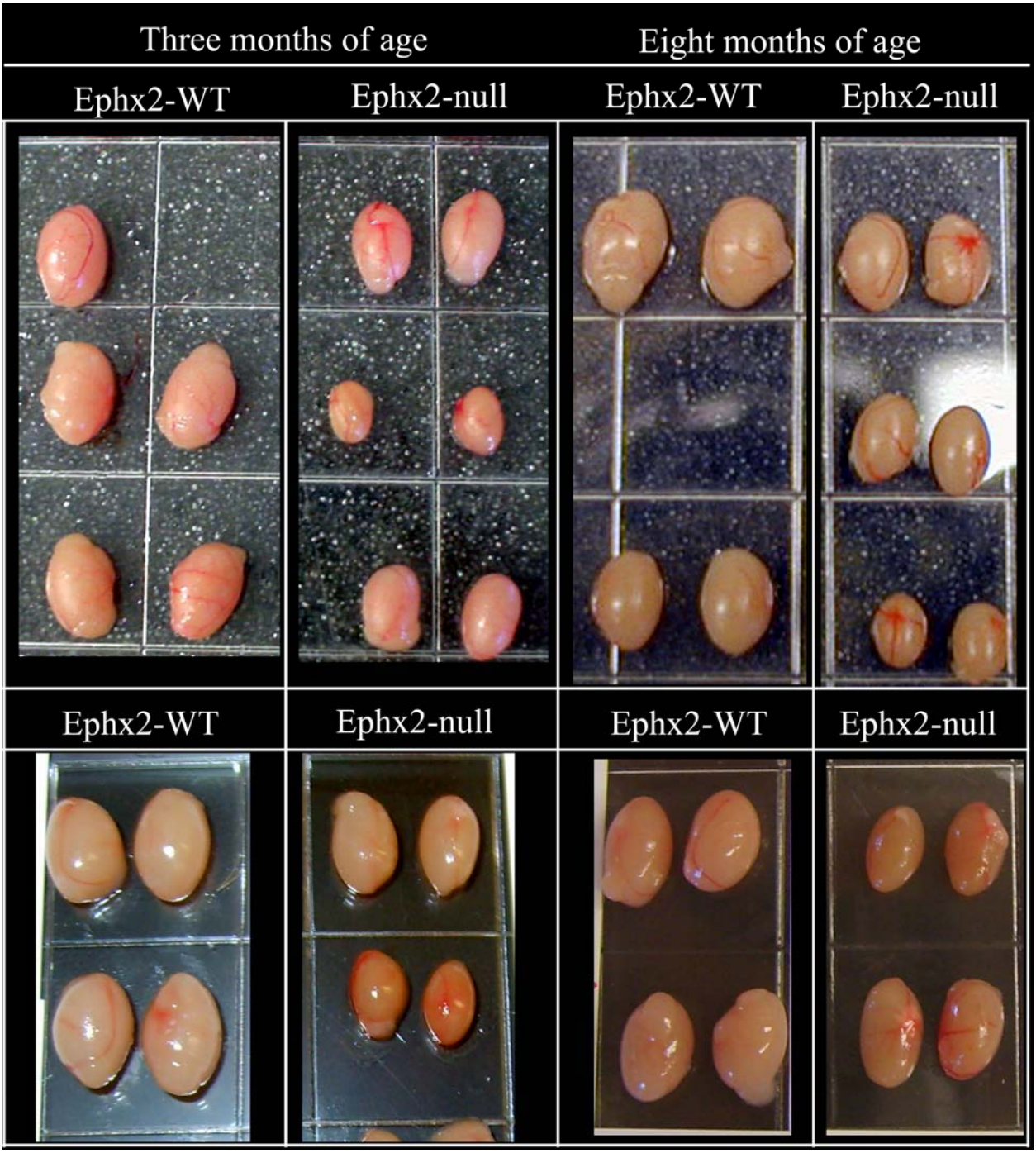

Three months of age
Eight months of age
Ephx2-WT
Ephx2-null
Ephx2-WT
Ephx2-null
Ephx2-WT
Ephx2-null
Ephx2-WT
Ephx2-null

Figure S1 B.

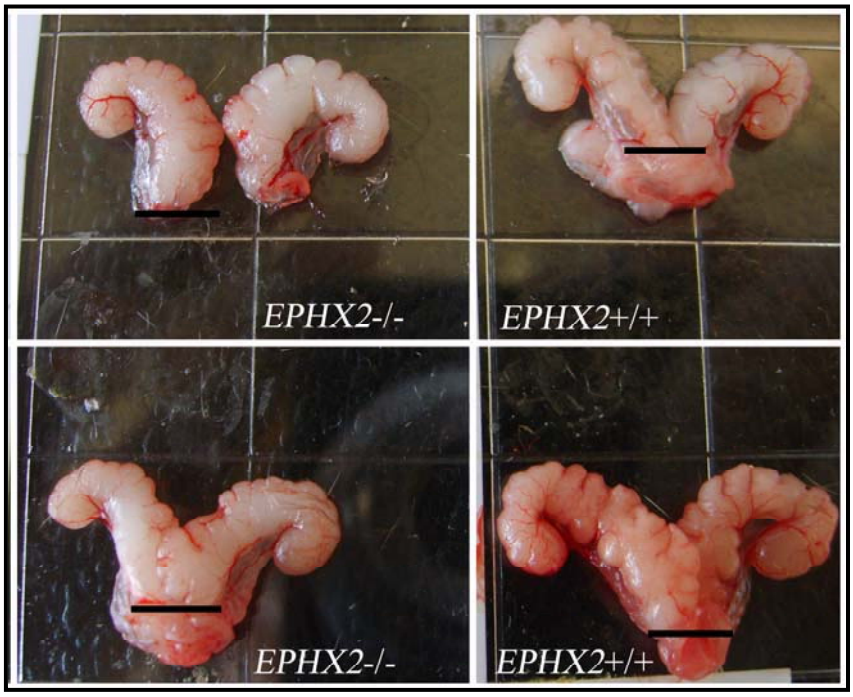


Figure S2.

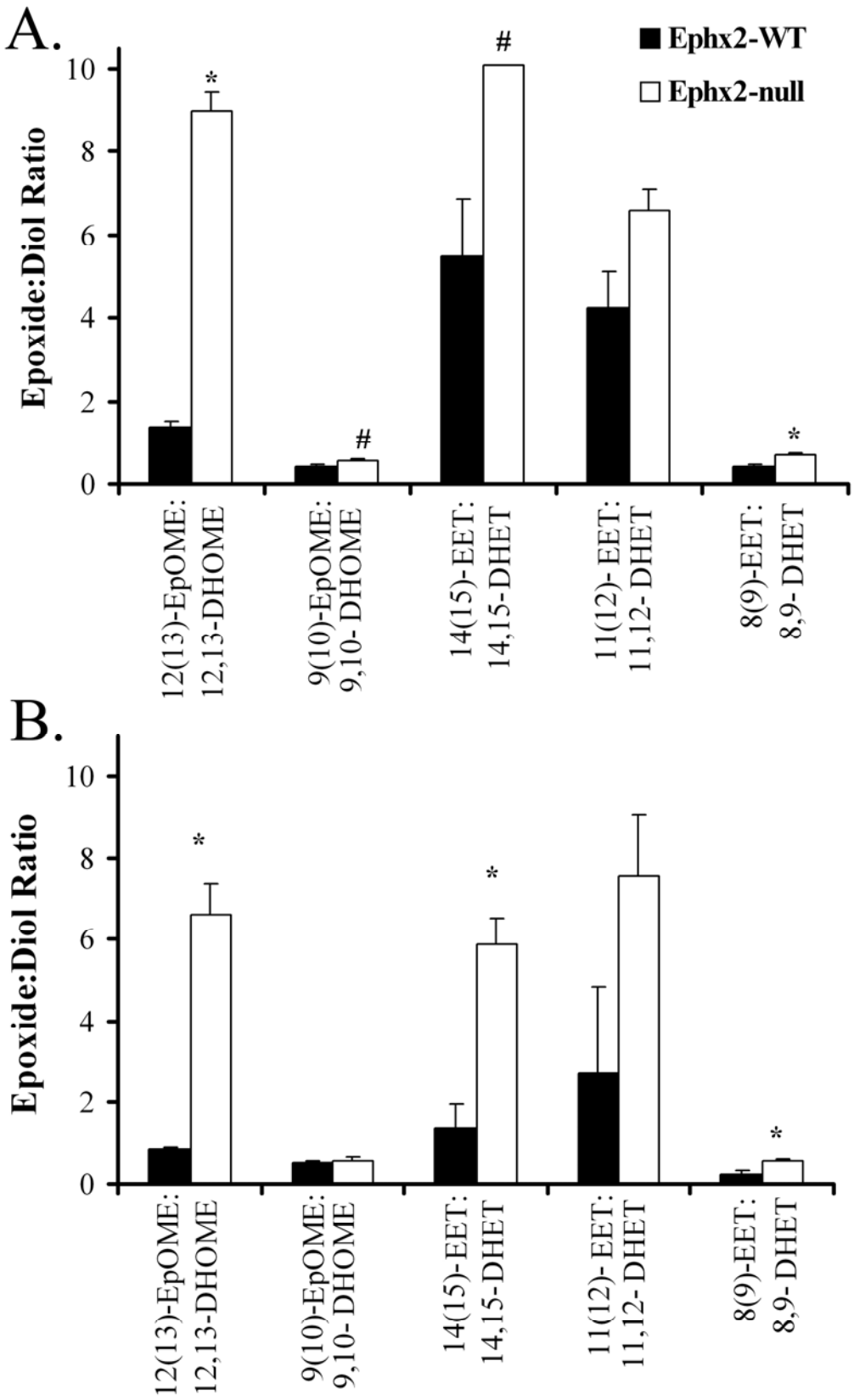
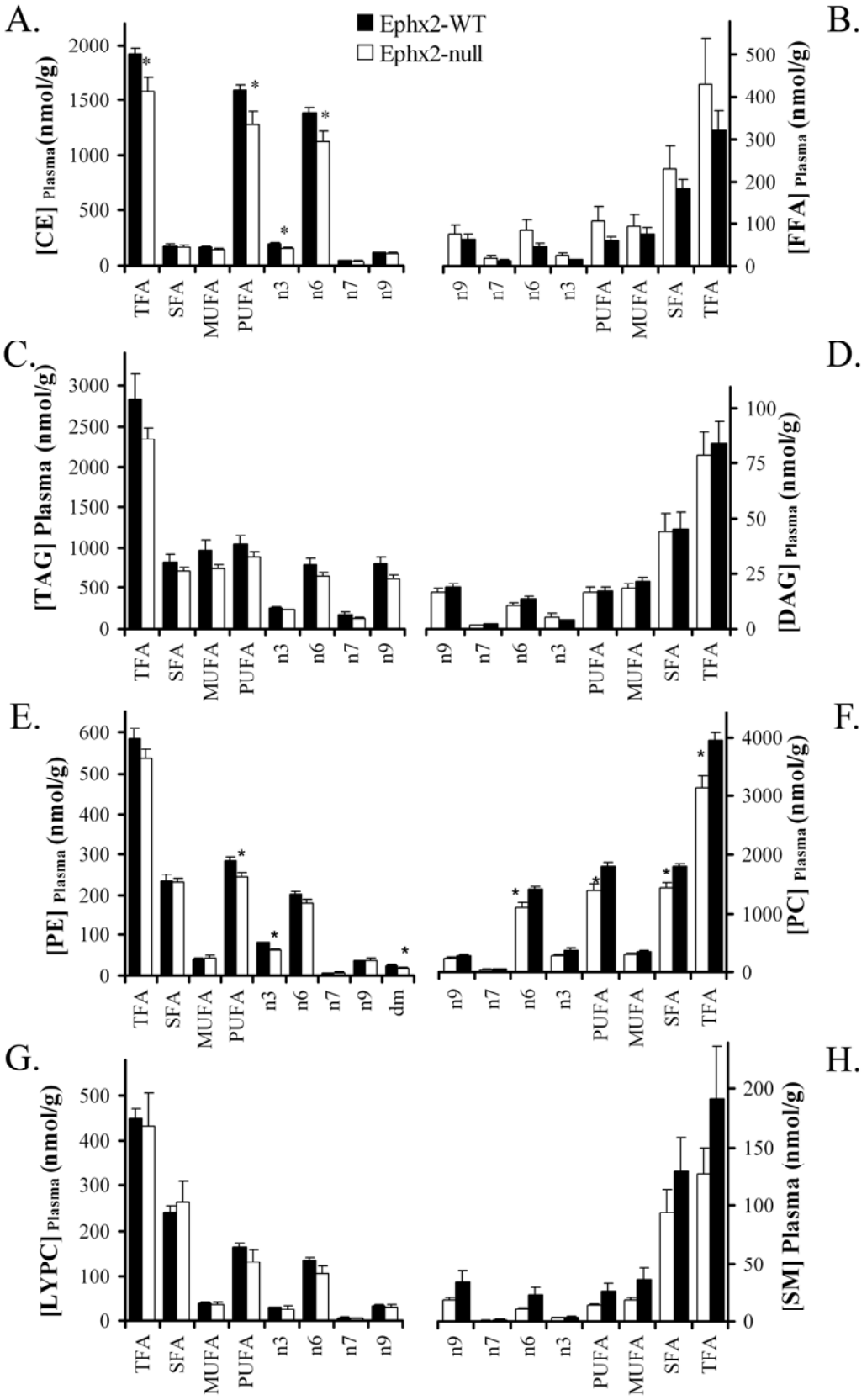

A.
Ephx2-WT
Ephx2-null
Epoxide:Diol Ratio
0
2
4
6
8
10
*
#
#
*
12(13)-EpOME: 12,13-DHOME
9(10)-EpOME: 9,10- DHOME
14(15)-EET: 14,15-DHET
11(12)- EET: 11,12- DHET
8(9)-EET: 8,9- DHET
B.
Epoxide:Diol Ratio
0
2
4
6
8
10
*
*
*
12(13)-EpOME: 12,13-DHOME
9(10)-EpOME: 9,10- DHOME
14(15)-EET: 14,15-DHET
11(12)- EET: 11,12- DHET
8(9)-EET: 8,9- DHET

Figure S3.


A.
Ephx2-WT
Ephx2-null
[CE] Plasma (nmol/g)
B.
[FFA] Plasma (nmol/g)
C.
[TAG] Plasma (nmol/g)
D.
[DAG] Plasma (nmol/g)
E.
[PE] Plasma (nmol/g)
F.
[PC] Plasma (nmol/g)
G.
[LYPC] Plasma (nmol/g)
H.
[SM] Plasma (nmol/g)
TFA
SFA
MUFA
PUFA
n3
n6
n7
n9
dm

Movies S1

A- Open field activity *Ephx2*-WT mice
B- Open field activity *Ephx2*-null mice

Movies S2

A- Sweetened condensed milk solution intake *Ephx2*-WT mice
B- Sweetened condensed milk solution intake *Ephx2*-WT mice
C- Sweetened condensed milk solution intake *Ephx2*-null mice